BMJ Open

Intestinal microbiota is affected by Helicobacter pylori infection in Japanese adolescents aged 14 or 15 years: a cross-sectional study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2020-047941
Article Type:	Original research
Date Submitted by the Author:	12-Dec-2020
Complete List of Authors:	kakiuchi, Toshihiko; Saga University, Department of Pediatrics Tanaka, Yoshiki; Biofermin Pharmaceutical Co Ltd Ohno, Hiroshi; Biofermin Pharmaceutical Co Ltd Matsuo, Muneaki; Saga University, Department of Pediatrics Fujimoto, Kazuma; International University of Health and Welfare, Department of Gastroenterology
Keywords:	Paediatric gastroenterology < PAEDIATRICS, Gastrointestinal infections < GASTROENTEROLOGY, Diagnostic microbiology < INFECTIOUS DISEASES

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Title: Intestinal microbiota is affected by *Helicobacter pylori* infection in Japanese adolescents aged 14 or 15 years: a cross-sectional study

Short title: Microbiota and *H. pylori* in adolescents

Toshihiko Kakiuchi[1] *, Yoshiki Tanaka[2], Hiroshi Ohno[2], Muneaki Matsuo[1] and Kazuma Fujimoto[3]

[1]Department of Pediatrics, Faculty of Medicine, Saga University, Saga, Japan

[2] R&D Center, Biofermin Pharmaceutical Co., Ltd., Kobe, Japan

[3] Department of Gastroenterology, International University of Health and Welfare, Fukuoka, Japan.

***Corresponding author:**

Toshihiko Kakiuchi, M.D., Ph. D.

Department of Pediatrics, faculty of Medicine, Saga University, Saga, Japan

E-mail: kakiucht@cc.saga-u.ac.jp

ABSTRACT

Objective: The relationship between *Helicobacter pylori* and the intestinal microbiota has not been clearly demonstrated in children and/or adolescents. The present study aimed to evaluate the effects of *H. pylori* infection on the intestinal microbiota in adolescents using genetic analysis.

Design: a cross-sectional study

Setting and participants: We included subjects from a longitudinal project involving *H. pylori* screening and treatment of junior high school third-grade students (aged 14 or 15 years) in Saga Prefecture. The study included a control group (n = 79) who were negative for anti-*H. pylori* antibody in urine and a *H. pylori* group (n = 80) who were positive for anti-*H. pylori* antibody in urine and *H. pylori* antigen in stool specimens.

Interventions: The intestinal microbiota was evaluated in stool specimens using 16S rRNA gene/DNA/amplicon sequencing with next generation sequencing.

Primary and secondary outcome measures: Alpha, beta diversity and relative abundances within the bacterial composition at the genus level in the control and *H. pylori* groups

Results: As shown by the alpha diversity of the 16S rRNA gene/DNA/amplicon sequence data, the control group had lower microbial species richness with lower alpha diversity compared with the *H. pylori* group ($P < 0.001$). Beta diversity of the intestinal microbiota profile also differed between the two groups ($P < 0.01$). The relative

abundance of the *Prevotella* genus was higher in the *H. pylori* group ($P < 0.01$) concomitant with a gain in body mass index in the *H. pylori* group ($P < 0.01$) compared with the control group.

Conclusions: The intestinal microbiota is significantly affected by *H. pylori* infection in Japanese adolescents. Additionally, the prevalence of the *Prevotella* genus is concomitantly increased along with the body mass index in *H. pylori*-infected students.

Trial registration number: This study was registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (No. UMIN000028721).

Strengths and limitations of this study

>The most strength of this study is that the effect of *Helicobacter pylori* (*H. pylori*) infection on the intestinal microbiota had been clearly demonstrated in children.

> Because the participants were Japanese adolescents of almost the same age living in a single prefecture, it is presumed that there would be no major difference the two groups.

>This study evaluated the intestinal microbiota using feces specimens, of which may be different from the mucosal-associated microbiota.

>The effect of eradication of *H. pylori* on the intestinal microbiota could not be analyzed, because the eradication therapy is important for intestinal microbiota changes.

Keywords: *Prevotella* genus, 16S rRNA, body mass index, screening and treatment

Abbreviations: *Helicobacter pylori* = *H. pylori*, OTUs = operational taxonomic units, ANOVA = analysis of variance, PERMDISP = permutational analysis of multivariate dispersions

Word count: 2,765words

INTRODUCTION

Newborns are exposed to various bacteria that are present in the mother's resident microbiota and the external environment. Bacterial species that comprise the intestinal microbiota change in an age-dependent manner [1 2]. Development of the intestinal microbiota during infancy is affected by several factors, including the maternal resident microbiota [3 4], the method of nutrition for infants [5-7], delivery style [5 8 9], and the administration of antibiotics [3 10 11].

Sustained infection of *Helicobacter pylori* decreases and/or increases gastric acid secretion, which might affect the gastric microbiota in adults [12-14] and children [12 13]. Several reports have suggested that the intestinal microbiota is significantly affected by *H. pylori* infection [14 15]. The effect of *H. pylori* infection on the intestinal microbiota has been investigated in adults [15 16], but has not been clearly demonstrated in children.

Therefore, the present study aimed to examine junior high school students in Japan aged from 14 to 15 years to determine whether *H. pylori* infection changes the intestinal microbiota. We also examined how body mass index (BMI) affects the intestinal microbiota, in addition to *H. pylori* infection.

METHODS

2.1. Study design and subjects

The longitudinal project for *H. pylori* screening and treatment among junior high school third-grade students in Saga Prefecture started in 2016 with the aim of primary

prevention of stomach cancer [17]. Figure 1 shows a flowchart of the junior high school third-grade students in Saga Prefecture in 2017. Among 8519 junior high school students aged 14 or 15 years old, 7230 received a screening urinary test (RAPIRAN; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to detect anti-*H. pylori* immunoglobulin-G antibody by immunochromatography. The diagnostic sensitivity, specificity, negative predictive value, and positive predictive value of the urinary test have been reported to be 78.4%, 100%, 90.1%, and 100%, respectively [18]. A total of 6874 students tested negative for *H. pylori* with the urinary test and 79 of these students were randomly selected as the *H. pylori*-negative group (control group). Students who tested positive in the screening urinary test received an *H. pylori* stool antigen detection test (TESTMATE RAPID PYLORI ANTIGEN; Wakamoto Pharmaceutical Co., Ltd. Tokyo, Japan). Among 290 students who received the stool antigen test, 234 students were positive for *H. pylori* infection. Finally, 80 of these students were randomly selected as the *H. pylori*-positive group (*H. pylori* group). The exclusion criteria for the present study were as follows: i) students who had taken medications, including proton-pump inhibitors, H_2 receptor antagonists, antacids, probiotics, mucosal protective agents, and/or antibiotics within the 6 months prior to enrollment, ii) students who were in the outpatient hospital because of sickness, and iii) students who had undergone eradication therapy for *H. pylori*.

The microbiota distribution was compared between the control and *H. pylori* groups regarding alpha diversity, beta diversity, and the relative abundance of the intestinal microbiota. The effect of BMI (low: < 15, middle: 15 to 25, high: > 25) on the microbiota distribution in the two groups was examined.

2.2. Stool sample collection and bacterial DNA extraction from feces

Each participant collected a stool sample at home for the present study using a paper stool collector and tube that was pre-filled with 5 ml of stool DNA stabilizer. Samples were immediately stored at -20°C and then delivered to the project center within 1 day. Extraction of bacterial DNA was performed as described previously [19]. A total of 20 mg of feces were washed three times in 1.0 ml of PBS and centrifuged (14,000 × g). The pellets were resuspended in a solution containing 450 µl of extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0) and 50 µl of 10% sodium dodecyl sulfate. A total of 300 mg of glass beads (diameter, 0.1 mm) and 500 µl of buffer-saturated phenol were added to the suspension and vortexed vigorously. After centrifugation at 14,000 × g for 5 min, 400 µl of the supernatant was extracted by phenol–chloroform, and 250 µl of the supernatant was subjected to isopropanol precipitation. Finally, the DNA was suspended in 1.0 ml of Tris-EDTA buffer.

2.3. DNA sequence analysis

Meta-analysis of the bacterial 16S rDNA sequences in the feces was performed in accordance with a previously described method [20] with minor modifications. In brief, the V3–V4 region of 16S rDNA was amplified on a Veriti thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). The amplicon was purified using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). For multiplex sequencing, a polymerase chain reaction was performed with dual eight-base indices (Nextera XT Index kit, Illumina, CA, USA). After purification by AMPure XP beads, the purified barcoded library was quantified fluorometrically using a QuantiT PicoGreen ds DNA Assay Kit (Invitrogen, Paisley, UK) and pooled at the same volume. The library pool (10 pM) was spiked with 40% PhiX control DNA (10 pM). Sequencing was conducted on a MiSeq platform with MiSeq Reagent Kit v2 chemistry (Illumina).

2.4. Microbiota analysis

Removal of low-quality sequences and chimera sequences, construction of operational taxonomic units (OTUs), and taxonomy assignment were conducted using the Quantitative Insights Into Microbial Ecology pipeline (http://qiime.org/) [21]. In brief, 50,000 raw reads were randomly obtained from the sequence files for each sample and merged by fastq-join with the default setting. Consequently, sequence reads with an average quality value of < 25 were removed and then chimera-checked. Five thousand reliable sequence reads were randomly obtained for each sample and OTUs were

constructed by clustering with a 97% identity threshold. The representative reads of each OTU were then assigned to the 16S rRNA gene database using UCLUST with ≥ 97% identity [22]. Comparison of each taxon in the gut microbiota was conducted at the genus level. Beta diversity was estimated by computing the weighted and unweighted UniFrac distances between samples [23]. To compare the differences in the overall bacterial gut microbiota structure, principal co-ordinates analysis was applied to reduce the dimensionality of the resulting distance matrix. The Shannon index, observed OTUs, chao 1, and the abundance-based coverage estimator index were calculated to investigate the alpha diversity of the microbiota in the samples.

2.5. Statistical analysis

All statistical analyses were conducted with R statistical software (R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). Data are shown as mean ± SE. Statistical significance was set at $P < 0.05$. During the analyses of the gut microbiotas, statistical significance was determined by a Welch's t test with Benjamini–Hochberg correlation. The relative abundance data were non-normally distributed; however, we applied Welch's T-test because the Mann–Whitney U-test is reported to be less robust [24]. Beta diversity was analyzed using permutational analysis of multivariate dispersions (PERMDISP) for comparisons of gene similarity.

RESULTS

3.1. Student characteristics

A total of 159 students participated in this study. The students' characteristics are shown in Table 1. There were no significant differences in sex, age, BMI, birth delivery style, method of infant nutrition, or the prevalence of allergic disease between the groups. The ratio of nursery school graduates to kindergarten graduates was significantly higher in the *H. pylori* group than in the control group ($P < 0.001$).

3.2. Alpha and beta diversity in the control and *H. pylori* groups

Figure 2 shows the alpha diversity of the 16S rRNA gene/DNA/amplicon sequence data. The control group showed lower microbial species richness with lower alpha diversity compared with the *H. pylori* group. The observed species index, chao 1 index, and ACE index all showed significantly higher diversity in the *H. pylori* group compared with the control group ($P < 0.001$). The Shannon index was not significantly different between the two groups ($P = 0.054$).

Figure 3 shows the beta diversity of the 16S rRNA gene/DNA/amplicon sequence data. Two-dimensional principle coordinate analysis of the weighted and unweighted UniFrac distances of the 16S rRNA gene/DNA/amplicon sequence data showed that the majority of samples were clustered dependent on the *H. pylori* infection status. The

analysis of similarity showed that the differences were significant for the weighted UniFrac distance ($P < 0.001$), but no significant for the unweighted UniFrac distance ($P = 0.643$) using PERMDISP.

3.3. Relative abundances within the bacterial composition at the genus level for the two groups

Figure 4 shows the 13 main types of bacteria present in the intestinal microbiota at the genus level as follows: *Bacteroides*, *Blautia*, *Bifidobacterium*, *Faecalibacterium*, *Prevotella*, *Fusicatenibacter*, *Eubacterium*, *Anaerostipes*, *Subdoligranulum*, *Streptococcus*, *Megamonas*, *Collinsella*, and *Clostridium*. The relative abundances of the *Prevotella* genus ($P < 0.01$) and *Collinsella* genus ($P < 0.05$) were significantly higher in the *H. pylori* group than in the control group. The relative abundance of the *Subdoligranulum* genus was significantly higher in the control group than in the *H. pylori* group ($P < 0.01$).

3.4. BMI and the relative abundances within the bacterial composition at the genus level

In the control and *H. pylori* groups, the intestinal microbiota was evaluated in association with BMI. Figure 5 shows the seven main types of bacteria in the intestinal microbiota at the genus level for the control group and the *H. pylori* group, categorized

by BMI. For the control group, these included *Bacteroides*, *Blautia*, *Bifidobacterium*, *Prevotella, Faecalibacterium*, *Fusicatenibacter*, and *Megamonas*. For the *H. pylori* group, these included *Prevotella, Bacteroides*, *Blautia*, *Bifidobacterium*, *Faecalibacterium*, *Megamonas*, and *Fusicatenibacter*. In the *H. pylori* group, the relative abundance of the *Prevotella* genus was significantly higher in the high BMI group compared with the middle and low BMI groups (both $P < 0.01$). Furthermore, the relative abundance of the *Prevotella* genus in the middle BMI group was higher than that in the low BMI group ($P < 0.05$). The relative abundances of *Bacteroides* and *Bifidobacterium* were significantly lower in the high BMI group compared with the other two groups (both $P < 0.05$). In the *H. pylori* group, BMI had no effect on the relative abundances of *Blautia*, *Faecalibacterium*, *Magamonas*, and *Fusicatenibacter*. In the control group, the relative abundance of the *Prevotella* genus was not significantly higher in the high BMI group compared with the middle and low BMI groups, whereas the relative abundance of the *Prevotella* genus significantly increased in proportion to an increase in BMI in the *H. pylori* group (low BMI vs high BMI: $P < 0.001$, middle BMI vs high BMI: $P < 0.001$) (Figure 6). The *Subdoligranulum* genus had a lower relative abundance in the high BMI category than in the low BMI group, but this trend was observed not only in the *H. pylori* group but also in the control group (Figure 7). The *Collinsella* genus was not associated with BMI regardless of *H. pylori* infection status (Figure 8).

DISCUSSION

The present study revealed two clinically important results: i) *H. pylori* infection significantly affected the intestinal microbiota of adolescents aged 14 or 15 years, as determined for Japanese junior high school students; ii) An increase in the relative abundance of the *Prevotella* genus in *H. pylori*-infected adolescents was concomitant with a gain in BMI.

Most reports of the effects of *H. pylori* on the intestinal microbiota based on the analysis of feces samples were in adults and data were lacking for children [25 26]. The present study showed a difference in the intestinal microbiota between *H. pylori*-infected and non-infected adolescents based on feces specimens. Alpha diversity, bacterial richness, and variance all showed greater diversity in *H. pylori*-infected students than in controls (Figure 2). A previous study showed that the diversity of the gastric microbiota in adolescents was enhanced by *H. pylori* infection [12]. Studies of the relationship between the intestinal microbiota and *H. pylori* infection are limited. One study reported a decrease in the *Firmicutes* genus in the human duodenal mucosa during *H. pylori* infection [27]. In the *H. pylori* infection model of Mongolian gerbils, the abundances of the *Bacteroides* and *Enterococcus* genera were increased in the duodenal mucosa [28]. In adults, the intestinal microbiota has been shown to be reduced in diversity during *H. pylori* infection [16] and our results were similar to those reported in adults

previously (Figure 2, 3). The human gut microbiota has been reported to form by the age of 3 years [29], so it may be that there is no difference in the effects of *H. pylori* infection on the intestinal microbiota between adolescents and adults.

It is known that infection with *H. pylori* reduces gastric acid secretion in children [30 31]. It was further suggested that a decrease in gastric acid secretion due to *H. pylori* infection may affect the intestinal flora of adolescents with *H. pylori* infection. In addition, a decrease in gastric acid secretion caused by *H. pylori* infection may allow a wide variety of bacteria in the oral cavity to more easily pass through the stomach and reach the lower gastrointestinal tract, thereby affecting the intestinal flora in feces. This might explain the result of the present study that alpha diversity of the fecal intestinal microbiota was increased in students with *H. pylori* infection. As suggested by the present study, *H. pylori* infection might be a factor that disturbs the intestinal microbiota in adolescents. The mechanisms and clinical importance of the effect of *H. pylori* warrant further investigation.

The *Prevotella* genus increased in abundance during *H. pylori* infection, and this increase was found to be concomitant with a rise in BMI in the present study. A previous report indicated that the *Prevotella* genus was elevated in abundance in school-age children infected with *H. pylori* [32]. The *Bacteroides* and *Bifidobacterium* genera are dominant among the intestinal microbiota in Japanese children [33]. A previous study showed that the prevalence rate of the *Prevotella* genus in the intestinal

microbiota was higher in subjects who consumed carbohydrates more frequently [34], which suggests that the *Prevotella* genus is closely related to eating habits. A correlation between *H. pylori* infection and the onset of diabetes has been reported in epidemiology studies [35 36], but the reason for this remains unknown. Meanwhile, the prevalence of the *Prevotella* genus increased in patients with obesity [37 38], nonalcoholic steatohepatitis [39], hyperlipidemia [40], and even in gestational diabetes, which is considered as a diabetes mellitus preliminary group [41]. The *Prevotella* genus is considered to contribute to hyperglycemia and insulin resistance [38 42 43]. In the present study, an increase in the relative abundance of *Prevotella* genus was observed in *H. pylori*-infected children with an increased BMI (Figure 5, 6). *H. pylori* infection in children with an elevated BMI without diabetes mellitus, caused an increase in the prevalence of the *Prevotella* genus (Figure 5, 6) and, as a result, insulin resistance increased, which may predispose individuals to diabetes mellitus. In fact, it is thought that the increase in *Prevotella* genus may be involved in the process of developing abnormal glucose metabolism as a result of obesity [44 45].

The *Subdoligranulum* genus showed a lower relative abundance in the high BMI category than in the low BMI group, but this trend was seen not only in the *H. pylori* group but also in the control group (Figure 7). The *Collinsella* genus was not associated with BMI regardless of *H. pylori* infection status (Figure 8). It has been reported that the *Subdoligranulum* genus is less prevalent among type 2 diabetes

patients compared with their non-diabetic counterparts [46], and a negative correlation with insulin resistance has been shown [47]. It has been reported that an increase in *Collinsella* genus is associated with an increase in insulin, triglyceride, and very low density lipoprotein levels [48], and is associated with type 2 diabetes [49]. In our study, of the three genera (*Prevotella, Subdoligranylum*, and *Collinsella*) that showed significant differences in relative abundance between the *H. pylori* and control groups, the *Prevotella* genus showed the most significant correlation between *H. pylori* infection status and BMI. The *Prevotella* genus was the only genus that showed an association with BMI in the *H. pylori* group but not the control group.

There are several limitations to the present study. i) The present study evaluated feces specimens, the microbiota of which may be different from the mucosal-associated microbiota. ii) The effect of eradication of *H. pylori* on the intestinal microbiota could be important [50], and we plan to investigate this in the future. iii) There was a difference in preschool status between the two groups (Table 1), and it could not be completely ruled out that this could have affected the intestinal microbiota.

CONCLUSION

The present study shows that the intestinal microbiota is significantly affected by *H. pylori* infection in junior high school third-grade students in Saga Prefecture, Japan.

Furthermore, the relative abundance of the *Prevotella* genus was increased concomitantly with a rise in BMI in *H. pylori*-infected students.

Acknowledgments

We would like to thank Mr. Daisuke Takami of R&D Center, Biofermin Pharmaceutical Co., Ltd. for his co-operation. We thank Ms. Kozue Kakiuchi, Ms. Tomomi Ito, and Ms. Hiromi Beppu for project support.

Author contributors

This study was supported by Biofermin Pharmaceutical Co., Ltd. (Kobe, Japan), who performed intestinal microbiota analysis and statistical evaluation. However, their contribution did not influence the analysis or interpretation of the data in this study. The authors (YT and HO) did not play any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Study concept and design: TK and KF. Acquisition of data: TK. Analysis and interpretation of data: TK. Drafting of the manuscript: TK. Critical revision of the manuscript for important intellectual content: MM and KF. Statistical analysis: YT and HO. Administrative, technical, or material support: YT and HO. Study supervision: MM and KF. Writing, reviewing, and editing: MM and KF.

Funding: This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests: The authors declare that they have no conflict of interest.

Patients consent for publication: Informed consent was obtained from all individual participants included in the study. Signed informed consent was obtained from each study participant prior to participation in the study.

Ethical approval: The ethical aspects of this study were reviewed and approved by the institutional review board of Saga University Hospital (approval number: 2016-11-03). Written informed consent was obtained from all of the students and their guardians. All methods were carried out in accordance with relevant guidelines and regulations or Helsinki guidelines.

Provenance and peer review: Not commissioned; externally peer-reviewed.

Date availability statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

REFERENCES

1 Matsuki T, Yahagi K, Mori H, *et al.* A key genetic factor for fucosyllactose utilization affects infant gut microbiota development. *Nat Commun* 2016;7:11939.

2 Yatsunenko T, Rey FE, Manary MJ, *et al.* Human gut microbiome viewed across age and geography. *Nature* 2012;486:222-7.

3 Romero R, Hassan SS, Gajer P, *et al.* The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2014;2:4.

4 Aagaard K, Riehle K, Ma J, *et al.* A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. *PLoS One* 2012;7:e36466.

5 Backhed F, Roswall J, Peng Y, *et al.* Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe* 2015;17:690-703.

6 Bezirtzoglou E, Tsiotsias A, Welling GW. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe* 2011;17:478-82.

7 Penders J, Vink C, Driessen C, *et al.* Quantification of Bifidobacterium spp., Escherichia coli and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett* 2005;243:141-7.

8 Bokulich NA, Chung J, Battaglia T, *et al.* Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci Transl Med* 2016;8:343ra82.

9 Mueller NT, Bakacs E, Combellick J, *et al.* The infant microbiome development: mom matters. *Trends Mol Med* 2015;21:109-17.

10 Fouhy F, Guinane CM, Hussey S, *et al.* High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrob Agents Chemother* 2012;56:5811-20.

11 Tanaka S, Kobayashi T, Songjinda P, *et al.* Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunol Med Microbiol* 2009;56:80-7.

12 Brawner KM, Kumar R, Serrano CA, *et al.* Helicobacter pylori infection is associated with an altered gastric microbiota in children. *Mucosal Immunol* 2017;10:1169-77.

13 Llorca L, Perez-Perez G, Urruzuno P, *et al.* Characterization of the Gastric Microbiota in a Pediatric Population According to Helicobacter pylori Status. *Pediatr Infect Dis J* 2017;36:173-8.

14 Oh B, Kim BS, Kim JW, *et al.* The Effect of Probiotics on Gut Microbiota during the Helicobacter pylori Eradication: Randomized Controlled Trial. *Helicobacter* 2016;21:165-74.

15 Buhling A, Radun D, Muller WA, *et al.* Influence of anti-Helicobacter triple-therapy with metronidazole, omeprazole and clarithromycin on intestinal microflora. *Aliment Pharmacol Ther* 2001;15:1445-52.

16 Kakiuchi T, Matsuo M, Endo H, *et al.* A Helicobacter pylori screening and treatment program to eliminate gastric cancer among junior high school students in Saga Prefecture: a preliminary report. *J Gastroenterol* 2019;54:699-707.

17 Kakiuchi T, Matsuo M, Endo H, *et al.* A Helicobacter pylori screening and treatment program to eliminate gastric cancer among junior high school students in Saga Prefecture: a preliminary report. *J Gastroenterol* 2019.

18 Okuda M, Kamiya S, Booka M, *et al.* Diagnostic accuracy of urine-based kits for detection of Helicobacter pylori antibody in children. *Pediatrics international : official journal of the Japan Pediatric Society* 2013;55:337-41.

19 Matsuki T, Watanabe K, Fujimoto J, *et al.* Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. *Appl Environ Microbiol* 2004;70:167-73.

20 Fadrosh DW, Ma B, Gajer P, *et al.* An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2014;2:6.

21 Caporaso JG, Kuczynski J, Stombaugh J, *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;7:335-6.

22 Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460-1.

23 Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 2005;71:8228-35.

24 Asaka M. A new approach for elimination of gastric cancer deaths in Japan. *Int J Cancer* 2013;132:1272-6.

25 Dash NR, Khoder G, Nada AM, *et al.* Exploring the impact of Helicobacter pylori on gut microbiome composition. *PLoS One* 2019;14:e0218274.

26 Yang YJ, Sheu BS. Metabolic Interaction of Helicobacter pylori Infection and Gut Microbiota. *Microorganisms* 2016;4.

27 Schulz C, Schutte K, Koch N, *et al.* The active bacterial assemblages of the upper GI tract in individuals with and without Helicobacter infection. *Gut* 2018;67:216-25.

28 Yin YN, Wang CL, Liu XW, *et al.* Gastric and duodenum microflora analysis after long-term Helicobacter pylori infection in Mongolian Gerbils. *Helicobacter* 2011;16:389-97.

29 Mitsuoka T, Hayakawa K, Kimura N. [The faecal flora of man. II. The composition of bifidobacterium flora of different age groups (author's transl)]. *Zentralbl Bakteriol Orig A* 1974;226:469-78.

30 Boukthir S, Aouididi F, Mazigh Mrad S, *et al.* [Chronic gastritis in children].

Tunis Med 2007;85:756-60.

31 Yu Y, Su L, Wang X, *et al.* Association between Helicobacter pylori infection and pathological changes in the gastric mucosa in Chinese children. *Intern Med* 2014;53:83-8.

32 Benavides-Ward A, Vasquez-Achaya F, Silva-Caso W, *et al.* Helicobacter pylori and its relationship with variations of gut microbiota in asymptomatic children between 6 and 12 years. *BMC Res Notes* 2018;11:468.

33 Nakayama J, Watanabe K, Jiang J, *et al.* Diversity in gut bacterial community of school-age children in Asia. *Sci Rep* 2015;5:8397.

34 Lim MY, Rho M, Song YM, *et al.* Stability of gut enterotypes in Korean monozygotic twins and their association with biomarkers and diet. *Sci Rep* 2014;4:7348.

35 Jeon CY, Haan MN, Cheng C, *et al.* Helicobacter pylori infection is associated with an increased rate of diabetes. *Diabetes Care* 2012;35:520-5.

36 Marietti M, Gasbarrini A, Saracco G, *et al.* Helicobacter pylori infection and diabetes mellitus: the 2013 state of art. *Panminerva Med* 2013;55:277-81.

37 Furet JP, Kong LC, Tap J, *et al.* Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers. *Diabetes* 2010;59:3049-57.

38 Moreno-Indias I, Sanchez-Alcoholado L, Garcia-Fuentes E, *et al.* Insulin

resistance is associated with specific gut microbiota in appendix samples from morbidly obese patients. *Am J Transl Res* 2016;8:5672-84.

39 Mouzaki M, Comelli EM, Arendt BM, *et al.* Intestinal microbiota in patients with nonalcoholic fatty liver disease. *Hepatology* 2013;58:120-7.

40 Roager HM, Licht TR, Poulsen SK, *et al.* Microbial enterotypes, inferred by the prevotella-to-bacteroides ratio, remained stable during a 6-month randomized controlled diet intervention with the new nordic diet. *Appl Environ Microbiol* 2014;80:1142-9.

41 Fugmann M, Breier M, Rottenkolber M, *et al.* The stool microbiota of insulin resistant women with recent gestational diabetes, a high risk group for type 2 diabetes. *Sci Rep* 2015;5:13212.

42 Pedersen HK, Gudmundsdottir V, Nielsen HB, *et al.* Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* 2016;535:376-81.

43 Lin L, Wen ZB, Lin DJ, *et al.* Correlations between microbial communities in stool and clinical indicators in patients with metabolic syndrome. *World J Clin Cases* 2018;6:54-63.

44 Murri M, Leiva I, Gomez-Zumaquero JM, *et al.* Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. *BMC Med* 2013;11:46.

45 Leiva-Gea I, Sanchez-Alcoholado L, Martin-Tejedor B, *et al.* Gut Microbiota

Differs in Composition and Functionality Between Children With Type 1 Diabetes and MODY2 and Healthy Control Subjects: A Case-Control Study. *Diabetes Care* 2018;41:2385-95.

46 Khorraminezhad L, Leclercq M, O'Connor S, *et al.* Dairy product intake modifies gut microbiota composition among hyperinsulinemic individuals. *Eur J Nutr* 2020.

47 Lv Y, Zhao X, Guo W, *et al.* The Relationship between Frequently Used Glucose-Lowering Agents and Gut Microbiota in Type 2 Diabetes Mellitus. *J Diabetes Res* 2018;2018:1890978.

48 Gomez-Arango LF, Barrett HL, McIntyre HD, *et al.* Connections Between the Gut Microbiome and Metabolic Hormones in Early Pregnancy in Overweight and Obese Women. *Diabetes* 2016;65:2214-23.

49 Lambeth SM, Carson T, Lowe J, *et al.* Composition, Diversity and Abundance of Gut Microbiome in Prediabetes and Type 2 Diabetes. *J Diabetes Obes* 2015;2:1-7.

50 Takara Y, Endo H, Nakano R, *et al.* Smoking and Drinking Did Not Increase the Failure of Therapeutic Helicobacter pylori Eradication by Vonoprazan, Clarithromycin, and Amoxicillin. *Digestion* 2019;99:172-8.

Table 1. Background characteristics of junior high school students in the two groups

		Control group (n = 79)	*H. pylori* group (n = 80)	*P* value
Sex	(male/female)	42/37	46/34	0.80
Age	(years)	14.73 ± 0.33	14.76 ± 0.32	0.71
BMI	(kg/m^2)	19.69 ± 3.48	19.67 ± 2.41	0.97
Delivery	(vaginal/C-section)	68/11	60/11	0.79
Nutrition	(breast/formula/mix)	37/6/36	27/15/36	0.07
School	(nursery/kindergarten/none)	25/54/0	53/25/2	< 0.001
Allergies	(+/−)	5/75	7/73	0.55

Delivery: birth delivery style; C-section: cesarean section; nutrition: method of infant nutrition; school: pre-school situation; BMI: body mass index.

Figure legends

Figure 1. Flowchart for *Helicobacter pylori* screening and treatment of junior high school students in Saga Prefecture and the selection method used to obtain the two groups.

The *H. pylori* group comprised 80 students who tested positive for urinary anti-*H. pylori* immunoglobulin-G antibody, gave a positive stool antigen test, and consented to the study. The control group (n = 79) comprised those who tested negative for both tests.

Figure 2. Alpha diversity of the 16S rRNA sequences in the control and *H. pylori* groups

The control group showed lower microbial species richness compared with the *H. pylori* group. The observed species index (S. obs), chao 1 index, and abundance-based coverage estimator index all showed significantly higher diversity in the *H. pylori* group than in the control group (*$P < 0.001$). The Shannon index was not significantly different between the two groups ($P = 0.054$). OTUs: operational taxonomic units.

Figure 3. Beta diversity of the 16S rRNA/DNA/amplicon sequence data (control group vs *H. pylori* group)

PCO: principal coordinate analysis; PERMDISP: permutational analysis of multivariate dispersions.

Figure 4. The main 13 types of bacteria present in the intestinal microbiota at the genus level, comparing the *H. pylori* and control groups.

*$P < 0.05$; **$P < 0.01$.

Figure 5. The seven main types of bacteria present in the intestinal microbiota at the genus level for the control group (A) and the *H. pylori* group (B) in association with body mass index (BMI)

*$P < 0.05$; **$P < 0.01$. Low: BMI < 15; Mid: BMI of 15 to 25; High: BMI > 25.

Figure 6. Relative abundance of the *Prevotella* genus in relation to BMI category in the *H. pylori* and control groups

Low: BMI < 15; Mid: BMI of 15 to 25; High: BMI > 25.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

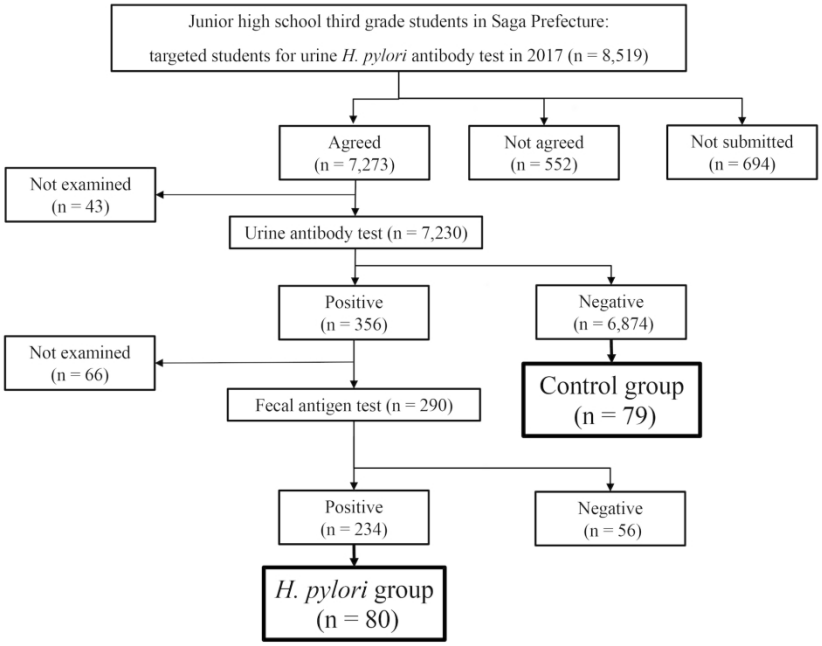
Figure 7. Relative abundance of the *Subdoligranulum* genus in relation to BMI category in the *H. pylori* and control groups

Low: BMI < 15; Mid: BMI of 15 to 25; High: BMI > 25.

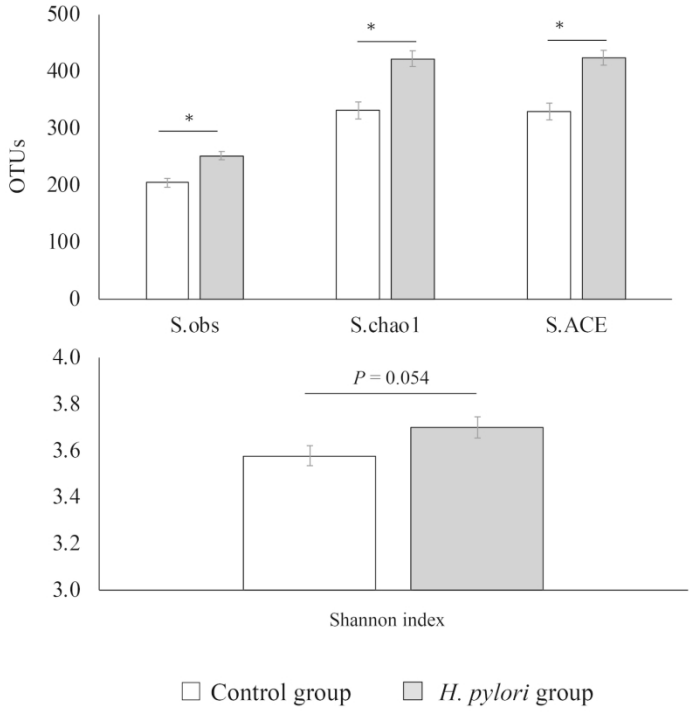
*$P < 0.05$; **$P < 0.01$.

Figure 8. Relative abundance of the *Collinsella* genus in relation to BMI category in the *H. pylori* and control groups

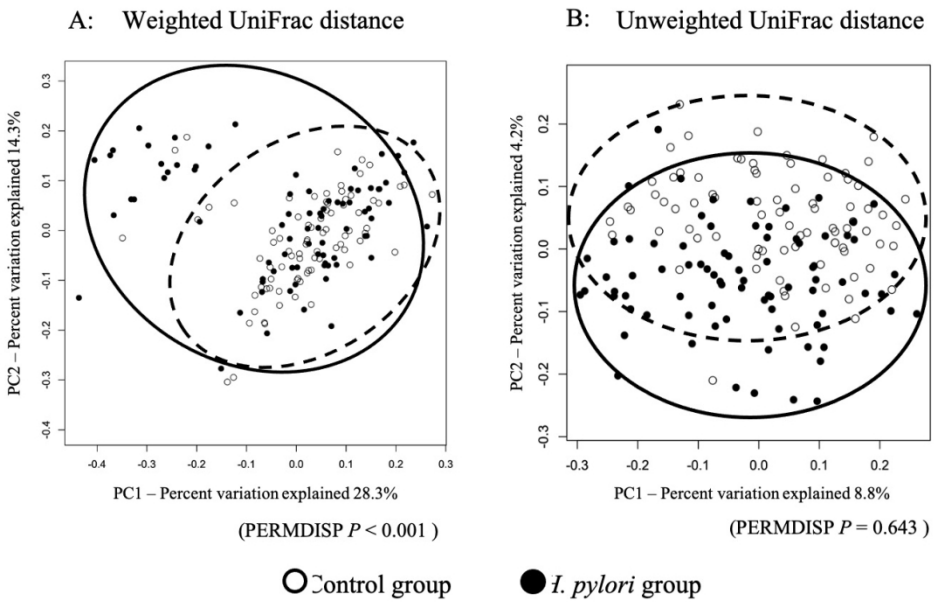
Low: BMI < 15; Mid: BMI of 15 to 25; High: BMI > 25.



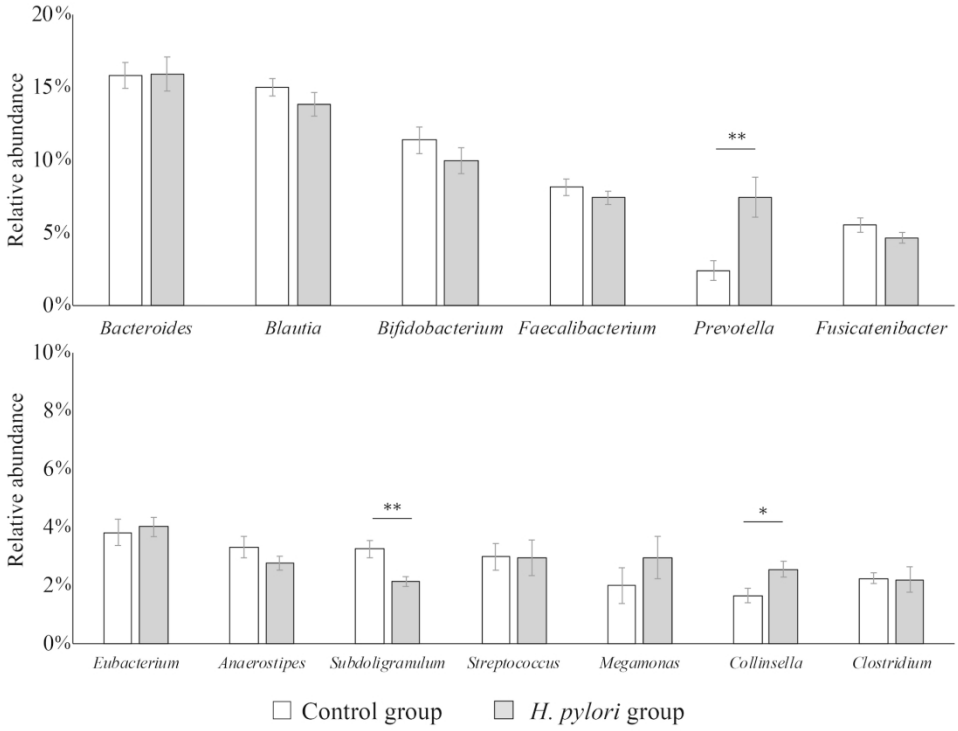
190x142mm (300 x 300 DPI)



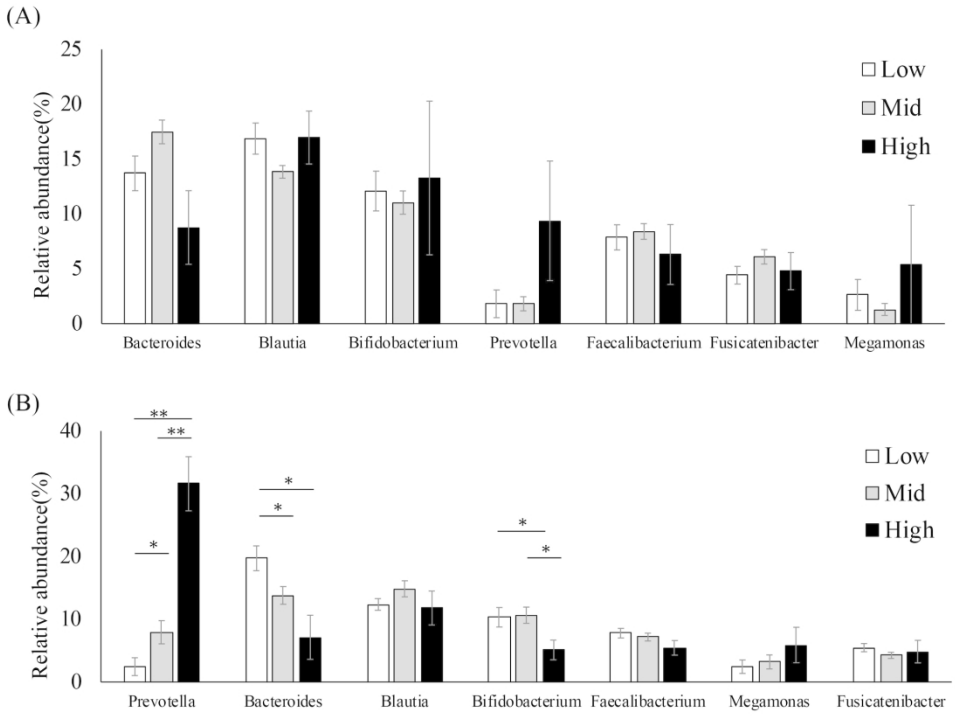
190x142mm (300 x 300 DPI)



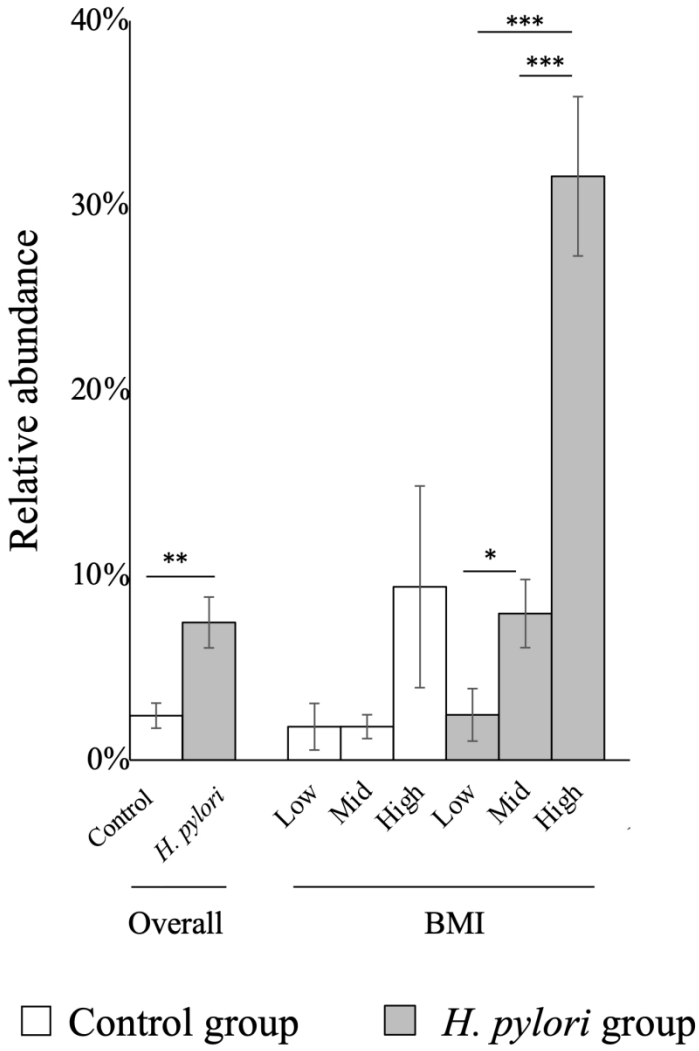
246x178mm (144 x 144 DPI)



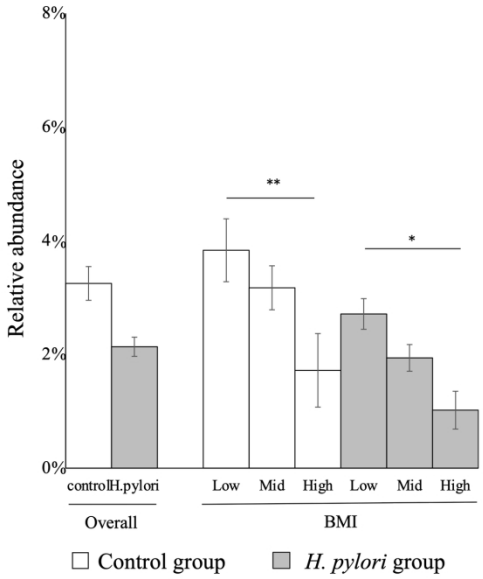
190x142mm (300 x 300 DPI)



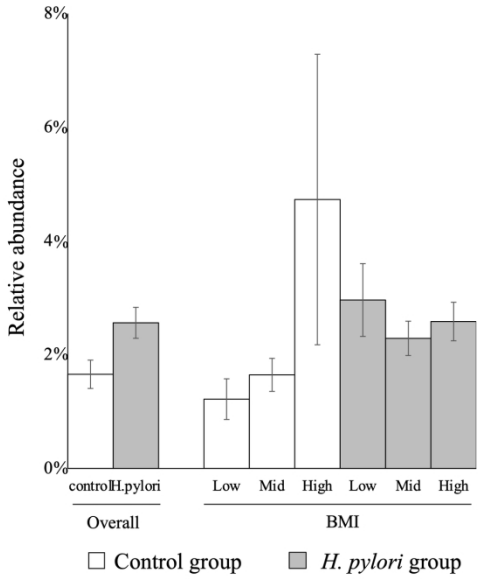
190x142mm (300 x 300 DPI)



159x197mm (300 x 300 DPI)



190x134mm (300 x 300 DPI)



190x134mm (300 x 300 DPI)

Reporting checklist for cross sectional study.

Based on the STROBE cross sectional guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the STROBE cross sectionalreporting guidelines, and cite them as:

von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.

		Reporting Item	Page Number
Title and abstract			
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	1
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	2-3
Introduction			
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	#3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	#4	Present key elements of study design early in the paper	5-6
Setting	#5	Describe the setting, locations, and relevant dates, including periods of	5-6

		recruitment, exposure, follow-up, and data collection	
Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of selection of participants.	6
	#7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-9
Data sources / measurement	#8	For each variable of interest give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group. Give information separately for for exposed and unexposed groups if applicable.	6-9
Bias	#9	Describe any efforts to address potential sources of bias	6-9
Study size	#10	Explain how the study size was arrived at	6-9
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	6-9
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding	9
Statistical methods	#12b	Describe any methods used to examine subgroups and interactions	9
Statistical methods	#12c	Explain how missing data were addressed	9
Statistical methods	#12d	If applicable, describe analytical methods taking account of sampling strategy	9
Statistical methods	#12e	Describe any sensitivity analyses	9
Results			
Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.	10
Participants	#13b	Give reasons for non-participation at each stage	10
Participants	#13c	Consider use of a flow diagram	10

Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	10
Descriptive data	#14b	Indicate number of participants with missing data for each variable of interest	10
Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	10-12
Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10-12
Main results	#16b	Report category boundaries when continuous variables were categorized	10-12
Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	10-12
Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	10-12
Discussion			
Key results	#18	Summarise key results with reference to study objectives	13
Limitations	#19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.	16
Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	13-16
Generalisability	#21	Discuss the generalisability (external validity) of the study results	13-16
Other Information			
Funding	#22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	18

BMJ Open

Helicobacter pylori infection-induced changes in the intestinal microbiota of 14 or 15 year-old Japanese adolescents: A cross-sectional study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2020-047941.R1
Article Type:	Original research
Date Submitted by the Author:	30-Apr-2021
Complete List of Authors:	kakiuchi, Toshihiko; Saga University, Department of Pediatrics Tanaka, Yoshiki; Biofermin Pharmaceutical Co Ltd Ohno, Hiroshi; Biofermin Pharmaceutical Co Ltd Matsuo, Muneaki; Saga University, Department of Pediatrics Fujimoto, Kazuma; International University of Health and Welfare, Department of Gastroenterology
Primary Subject Heading:	Gastroenterology and hepatology
Secondary Subject Heading:	Infectious diseases
Keywords:	Paediatric gastroenterology < PAEDIATRICS, Gastrointestinal infections < GASTROENTEROLOGY, Diagnostic microbiology < INFECTIOUS DISEASES

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Title: *Helicobacter pylori* infection-induced changes in the intestinal microbiota of 14 or 15 year-old Japanese adolescents: A cross-sectional study

Toshihiko Kakiuchi[1 *], Yoshiki Tanaka[2], Hiroshi Ohno[2], Muneaki Matsuo[1], and Kazuma Fujimoto[3]

[1]Department of Pediatrics, Faculty of Medicine, Saga University, Saga, Japan

[2] R&D Center, Biofermin Pharmaceutical Co., Ltd., Kobe, Japan

[3] Department of Gastroenterology, International University of Health and Welfare, Fukuoka, Japan.

***Corresponding author:**

Toshihiko Kakiuchi, M.D., Ph. D.

Department of Pediatrics, faculty of Medicine, Saga University, Saga, Japan

E-mail: kakiucht@cc.saga-u.ac.jp

Word count: 3,581

ABSTRACT

Objective: The relationship between *Helicobacter pylori* and the intestinal microbiota has not yet been clearly demonstrated in children and adolescents. The present study aimed at evaluating how *H. pylori* infection could affect the intestinal microbiota in adolescents using genetic analysis.

Design: cross-sectional study

Setting and participants: We included subjects from a longitudinal project involving *H. pylori* screening and treatment of junior high school third-grade students (aged 14 or 15 years) in Saga Prefecture. The study included a control group (n = 79) and an *H. pylori* group (n = 80) tested negative and positive for the anti-*H. pylori* antibody in the urine and *H. pylori* antigen in stool specimens, respectively.

Interventions: The intestinal microbiota was evaluated in stool specimens using 16S rRNA gene/DNA/amplicon sequencing with next-generation sequencing.

Primary and secondary outcome measures: We assessed alpha and beta diversity, just as well as relative abundances within the bacterial composition at the genus level in both groups.

Results: As shown by the alpha diversity of the 16S rRNA gene/DNA/amplicon sequence data, the control group exhibited lower microbial species richness with lower

alpha diversity compared with the *H. pylori* group ($P < 0.001$). The beta diversity of the intestinal microbiota profile also differed between the two groups ($P < 0.01$). The relative abundance of the *Prevotella* genus was higher in the *H. pylori* group ($P < 0.01$) concomitant with a gain in body mass index in the *H. pylori* group ($P < 0.01$) compared with the control group.

Conclusions: *H. pylori* infection significantly affected the intestinal microbiota in Japanese adolescents. In addition, the prevalence of the *Prevotella* genus is concomitantly increased along with the body mass index in *H. pylori*-infected students.

Trial registration number: This study was registered in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (No. UMIN000028721).

Strengths and limitations of this study

- The most significant strength of this study is that it clearly demonstrated the effect of *Helicobacter pylori* (*H. pylori*) infection on the intestinal microbiota of children.
- As the participants were Japanese adolescents of almost the same age living in a single prefecture, no major difference would presumably exist between the two groups.
- This study evaluated the intestinal microbiota using feces specimens, which might differ from the mucosal-associated microbiota

- The effect of *H. pylori* eradication on the intestinal microbiota could not be analyzed, as the eradication therapy is important for intestinal microbiota changes.

Keywords: *Prevotella* genus, 16S rRNA, body mass index, screening and treatment

Abbreviations: *Helicobacter pylori* = *H. pylori*, proton-pump inhibitors = PPIs, OTUs = operational taxonomic units, ANOVA = analysis of variance, PERMDISP = permutational analysis of multivariate dispersions, F/B ration = ratio of *Firmicutes* phylum to *Bacteroides* phylum

INTRODUCTION

Newborns are exposed to various bacteria that are present in the mother's resident microbiota and the external environment. Bacterial species that comprise the intestinal microbiota change in an age-dependent manner [1, 2]. The development of the intestinal microbiota during infancy is affected by several factors, including the maternal resident microbiota [3, 4], the method of nutrition for infants [5-7], delivery style [5, 8, 9], and the administration of antibiotics [3, 10, 11].

The global *H. pylori* prevalence in children varies significantly, from 2.5% in Japan to 34.6% in Ethiopia [12]. Sustained infection of *Helicobacter pylori* decreases or increases gastric acid secretion, which might affect the gastric microbiota in adults [13-15] and children [13, 14]. Several previous reports have suggested that the intestinal microbiota is significantly affected by *H. pylori* infection [15, 16]. The effect of *H. pylori* infection on the intestinal microbiota has been demonstrated in adults [16, 17] but has not been fully investigated in children.

Therefore, the present study aimed at examining junior high school students in Japan aged 14–15 years to determine whether *H. pylori* infection changes the intestinal microbiota. Moreover, we also examined how body mass index (BMI) affects the intestinal microbiota, in addition to *H. pylori* infection.

METHODS

2.1. Study design and subjects

The longitudinal project for *H. pylori* screening and treatment among junior high school third-grade students in Saga Prefecture started in 2016 with the aim of primary prevention of stomach cancer [17]. Figure 1 shows a flowchart of the junior high school third-grade students in Saga Prefecture in 2017. Among 8519 junior high school students aged 14 or 15 years, 7230 received a screening urinary test (RAPIRAN; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to detect anti-*H. pylori* immunoglobulin-G antibody by immunochromatography. There is an established screening program for kidney diseases in Saga Prefecture, targeting third-grade students in junior high schools. Given the full inclusivity of student during this test through simple urine examination, we used the established system to obtain urine samples to screen for *H. pylori* infection [17]. The diagnostic sensitivity, specificity, negative predictive value, and positive predictive value of the urinary test was reportedly 78.4, 100, 90.1, and 100%, respectively [18]. A total of 6874 students tested negative for *H. pylori* with the urinary test and 79 of these students were randomly selected as the *H. pylori*-negative group (control group). Students who tested positive in the screening urinary test received an

H. pylori stool antigen detection test (TESTMATE RAPID PYLORI ANTIGEN; Wakamoto Pharmaceutical Co., Ltd. Tokyo, Japan). Among 290 students who received the stool antigen test, 234 students tested positive for *H. pylori* infection. Finally, 80 of these students were randomly selected as the *H. pylori*-positive group (*H. pylori* group). The exclusion criteria for the present study were as follows: i) students who had taken medications, including proton-pump inhibitors (PPIs), H_2 receptor antagonists, antacids, probiotics, mucosal protective agents, and antibiotics within the 6 months prior to enrollment, ii) students who were in the outpatient hospital because of sickness, and iii) students who had undergone eradication therapy for *H. pylori*.

The microbiota distribution was compared between the control and *H. pylori* groups regarding alpha diversity, beta diversity, and the relative abundance of the intestinal microbiota. The effect of BMI (low: <15, middle: 15–25, high: >25) on the microbiota distribution in the two groups was examined.

2.2. Stool sample collection and bacterial DNA extraction from feces

Each participant collected a stool sample at home for the present study using a paper stool collector and tube that was pre-filled with 5 ml of a stool DNA stabilizer. The stool collection method was performed according to the attached document of the stool

collection kit. Samples were immediately stored at −20 °C and delivered to the project center within a day. Extraction of bacterial DNA was performed as described previously [19]. A total of 20 mg of feces was washed three times in 1.0 ml of PBS and centrifuged (14,000 × g). The pellets were resuspended in a solution containing 450 µl of extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0) and 50 µl of 10% sodium dodecyl sulfate. A total of 300 mg of glass beads (diameter, 0.1 mm) and 500 µl of buffer-saturated phenol were added to the suspension and vortexed vigorously. After centrifugation at 14,000 × g for 5 min, 400 µl of the supernatant was extracted by phenol-chloroform, and 250 µl of the supernatant was subjected to isopropanol precipitation. Finally, the DNA was suspended in 1.0 ml of Tris-EDTA buffer.

2.3. DNA sequence analysis

We performed the meta-analysis of the bacterial 16S rDNA sequences in the feces in accordance with a previously described method [20] with minor modifications. Briefly, the V3–V4 region of 16S rDNA were amplified on a Veriti thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). The amplicon was purified using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). For multiplex sequencing, a polymerase chain reaction was performed with dual eight-base indices (Nextera XT

Index kit, Illumina, CA, USA). After purification by AMPure XP beads, the purified barcoded library was quantified fluorometrically using a QuantiT PicoGreen ds DNA Assay Kit (Invitrogen, Paisley, UK) and pooled at the same volume. The library pool (10 pM) was spiked with 40% PhiX control DNA (10 pM). Sequencing was conducted on a MiSeq platform with MiSeq Reagent Kit v2 chemistry (Illumina).

2.4. Microbiota analysis

We conducted the removal of low-quality and chimera sequences, construction of operational taxonomic units (OTUs), and taxonomy assignment using the Quantitative Insights Into Microbial Ecology pipeline (http://qiime.org/) [21]. Briefly, 50,000 raw reads were randomly obtained from the sequence files for each sample and merged by fastq-join with the default setting. Consequently, sequence reads with an average quality value of <25 were removed and then chimera-checked. Five thousand reliable sequence reads were randomly obtained for each sample and OTUs were constructed by clustering with a 97% identity threshold. The representative reads of each OTU were then assigned to the 16S rRNA gene database using UCLUST with ≥ 97% identity [22]. A comparison of each taxon in the gut microbiota was conducted at the genus level. Beta diversity was estimated by computing the weighted and unweighted UniFrac

distances between the samples [23]. In order to compare the differences in the overall bacterial gut microbiota structure, principal coordinates analysis was applied to reduce the dimensionality of the resulting distance matrix. We calculated the Shannon index, observed OTUs, chao 1, and the abundance-based coverage estimator index to investigate the alpha diversity of the microbiota in the samples.

2.5. Statistical analysis

All statistical analyses were conducted with the R statistical software (R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). Data are shown as the mean ± SE. Statistical significance was set at $P < 0.05$. During the analyses of the gut microbiotas, the statistical significance was determined by Welch's t-test with Benjamini–Hochberg correlation. The relative abundance data were non-normally distributed. However, we applied Welch's t-test as the Mann–Whitney U-test is reportedly less robust [24]. Beta diversity was analyzed using permutational analysis of multivariate dispersions (PERMDISP) for comparisons of gene similarity.

2.6. Patient and Public Involvement

This study was performed without patient involvement. Patients were not invited to comment on the study design and were not consulted to develop patient-relevant outcomes or interpret the results. Patients were not invited to contribute to the writing or editing of the manuscript for readability or accuracy.

RESULTS

3.1. Student characteristics

A total of 159 students participated in this study. The student characteristics are shown in Table 1. No significant differences could be observed in sex, age, BMI, birth delivery style, method of infant nutrition, or the prevalence of allergic disease between the groups. The ratio of nursery school graduates to kindergarten graduates was significantly higher in the *H. pylori* group than in the control group ($P < 0.001$). The subjects of this study did not include low-birth-weight infants (birth weight 2500 g or less). In addition, we did not investigate whether symptoms associated with *H. pylori* infection, such as abdominal symptoms, were present in the *H. pylori* group.

3.2. Alpha and beta diversity in the control and the *H. pylori* groups

Figure 2 shows the alpha diversity of the 16S rRNA gene/DNA/amplicon sequence data. The control group showed lower microbial species richness with lower alpha diversity compared with the *H. pylori* group. The observed species index, chao 1 index, and ACE index all showed significantly higher diversity in the *H. pylori* group compared with the control group ($P < 0.001$). The Shannon index was not significantly different between the two groups ($P = 0.054$).

Figure 3 shows the beta diversity of the 16S rRNA gene/DNA/amplicon sequence data. The two-dimensional principal coordinate analysis of the weighted and unweighted UniFrac distances of the 16S rRNA gene/DNA/amplicon sequence data showed that the majority of samples were clustered dependent on the *H. pylori* infection status. The similarity analysis showed that the differences were significant for the weighted UniFrac distance ($P < 0.001$), but not for the unweighted UniFrac distance ($P = 0.643$) using PERMDISP.

3.3. Relative abundances within the bacterial composition at the genus level for the two groups

Figure 4 shows the 13 main bacterial types present in the intestinal microbiota at the genus level as follows: *Bacteroides*, *Blautia*, *Bifidobacterium*, *Faecalibacterium*,

Prevotella, *Fusicatenibacter*, *Eubacterium*, *Anaerostipes*, *Subdoligranulum*, *Streptococcus*, *Megamonas*, *Collinsella*, and *Clostridium*. The relative abundances of the *Prevotella* genus ($P < 0.01$) and *Collinsella* genus ($P < 0.05$) were significantly higher in the *H. pylori* group than in the control group. The relative abundance of the *Subdoligranulum* genus was significantly higher in the control group than in the *H. pylori* group ($P < 0.01$). At the phylum level, the ratio of the *Firmicutes* to the *Bacteroides* phyla (F/B ratio) showed no significant difference between the two groups (the control group; 4.19 ± 3.27 vs. the *H. pylori* group; 4.87 ± 12.04, $P = 0.63$).

3.4. BMI and the relative abundances within the bacterial composition at the genus level

In the control and *H. pylori* groups, the intestinal microbiota was evaluated in association with the BMI. Figure 5 shows the seven main bacterial types in the intestinal microbiota at the genus level for the control and the *H. pylori* groups, categorized by the BMI. For the control group, these included *Bacteroides*, *Blautia*, *Bifidobacterium*, *Prevotella, Faecalibacterium*, *Fusicatenibacter*, and *Megamonas* and for the *H. pylori* group *Prevotella, Bacteroides*, *Blautia*, *Bifidobacterium*, *Faecalibacterium*, *Megamonas*, and *Fusicatenibacter*. In the *H. pylori* group, the

relative abundance of the *Prevotella* genus was significantly higher in the high-BMI group compared with the middle- and low-BMI groups (both $P < 0.01$). Furthermore, the relative abundance of the *Prevotella* genus in the middle-BMI group was higher than that in the low-BMI group ($P < 0.05$). The relative abundances of *Bacteroides* and *Bifidobacterium* were significantly lower in the high-BMI group compared with the other two groups (both $P < 0.05$). In the *H. pylori* group, the BMI did not affect the relative abundances of *Blautia*, *Faecalibacterium*, *Magamonas*, and *Fusicatenibacter*. In the control group, the relative abundance of the *Prevotella* genus was not significantly higher in the high-BMI group compared with the middle- and low-BMI groups, whereas the relative abundance of the *Prevotella* genus significantly and proportionately increased with an increasing BMI in the *H. pylori* group (low BMI vs high BMI: $P < 0.001$, middle BMI vs high BMI: $P < 0.001$) (Figure 6). At the phylum level, we observed no significant differences in the F/B ratio among the three BMI categories in the control groups. However, a significant difference could be detected between the high and middle BMI categories in the *H. pylori* group (Figure 7). The *Subdoligranulum* genus had a lower relative abundance in the high-BMI category than in the low-BMI group, although this trend was observed not only in the *H. pylori* group

but also in the control group (Figure 8). The *Collinsella* genus was not associated with the BMI regardless of *H. pylori* infection status (Figure 9).

DISCUSSION

The present study revealed two clinically important results: i) *H. pylori* infection significantly affected the intestinal microbiota of adolescents aged 14 or 15 years, as determined for Japanese junior high school students; ii) An increase in the relative abundance of the *Prevotella* genus in *H. pylori*-infected adolescents was concomitant with a gain in BMI.

Most reports of the effects of *H. pylori* on the intestinal microbiota based on the analysis of feces samples were in adults and data were lacking for children [25, 26]. The present study showed a difference in the intestinal microbiota between *H. pylori*-infected and non-infected adolescents based on feces specimens. Alpha diversity, bacterial richness, and variance all showed greater diversity in *H. pylori*-infected students than in controls (Figure 2). A previous study showed that the diversity of the gastric microbiota in adolescents was enhanced by *H. pylori* infection [13]. Studies of the relationship between the intestinal microbiota and *H. pylori* infection are limited. One study reported a decrease in the *Firmicutes* genus in the human duodenal mucosa

during *H. pylori* infection [27]. In the *H. pylori* infection model of Mongolian gerbils, the abundances of the *Bacteroides* and *Enterococcus* genera were increased in the duodenal mucosa [28]. In adults, *H. pylori* infection [17] reportedly reduced intestinal microbiota diversity and our results were in good agreement with these previous reports (Figure 2, 3). The human gut microbiota has been reported to form by the age of 3 years [29], so it may be that there is no difference in the effects of *H. pylori* infection on the intestinal microbiota between adolescents and adults.

It is known that infection with *H. pylori* reduces gastric acid secretion in children [30, 31]. It was further suggested that a decrease in gastric acid secretion due to *H. pylori* infection may affect the intestinal flora of adolescents with *H. pylori* infection. In addition, a decrease in gastric acid secretion caused by *H. pylori* infection may allow a wide variety of bacteria in the oral cavity to more easily pass through the stomach and reach the lower gastrointestinal tract, thereby affecting the intestinal flora in feces. The inhibitory effect of PPIs on gastric acid secretion affects the composition of the intestinal flora. PPIs administration causes an increase in the indigenous bacteria the *Streptococcus* genus and the *Lactobacillus* genus in the intestine, which is thought to be due to the oral bacteria reaching the intestine to suppress gastric acid secretion [32, 33]. This might explain the result of the present study that alpha diversity of the fecal

intestinal microbiota was increased in students with *H. pylori* infection. As suggested by the present study, *H. pylori* infection might be a factor that disturbs the intestinal microbiota in adolescents. *H. pylori* infection is involved in the alterations of gut microbiota composition and diversity, which can lead to changes in production level and physiologic regulation of the gut metabolic hormones released from the host endocrine system [34]. The mechanisms and clinical importance of the effect of *H. pylori* warrant further investigation.

The *Prevotella* genus increased in abundance during *H. pylori* infection, and this increase was found to be concomitant with a rise in BMI in the present study. A previous report indicated that the *Prevotella* genus was elevated in abundance in school-age children infected with *H. pylori* [35]. This was an epidemiological study, and unfortunately, it is completely unknown why at this time the *Prevotella* genus is elevated in school-age children infected with *H. pylori*. The *Bacteroides* and *Bifidobacterium* genera are dominant among the intestinal microbiota in Japanese children [36]. A previous study showed that the prevalence rate of the *Prevotella* genus in the intestinal microbiota was higher in subjects who consumed carbohydrates more frequently [37], which suggests that the *Prevotella* genus is closely related to eating habits. In the present study, it is not possible to determine whether infection with *H.*

pylori affected the diet and resulted in an increase in the *Prevotella* genus, or whether infection with *H. pylori* increased the *Prevotella* genus and affected the diet, and caused an increase in BMI. In general, the F/B ratio has been found to increase with obesity [38]. The *Prevotella* genus belongs to the *Bacteroides* phylum, the present study thus exhibited inconsistency. At the moment, it is currently difficult to associate the F/B ratio with a determined health status and, more specifically, to consider it as a hallmark of obesity [39]. In the future, regarding the relationship between *H. pylori* and the *Prevotella* genus and BMI, it is necessary to analyze the intestinal flora in early childhood, including the history of eating habits from early childhood.

A correlation between *H. pylori* infection and the onset of diabetes has been reported in epidemiology studies [40, 41], but the reason for this remains unknown. Meanwhile, the prevalence of the *Prevotella* genus increased in patients with obesity [42, 43], nonalcoholic steatohepatitis [44], hyperlipidemia [45], and even in gestational diabetes, which is considered as a diabetes mellitus preliminary group [46]. The *Prevotella* genus is considered to contribute to hyperglycemia and insulin resistance [43, 47, 48]. In the present study, an increase in the relative abundance of the *Prevotella* genus was observed in *H. pylori*-infected children with an increased BMI (Figure 5, 6). *H. pylori* infection in children with an elevated BMI without diabetes mellitus, caused

an increase in the prevalence of the *Prevotella* genus (Figure 5, 6) and, as a result, insulin resistance increased, which may predispose individuals to diabetes mellitus. In fact, it is thought that the increase in *Prevotella* genus may be involved in the process of developing abnormal glucose metabolism as a result of obesity [49, 50].

The *Subdoligranulum* genus showed a lower relative abundance in the high BMI category than in the low BMI group, but this trend was seen not only in the *H. pylori* group but also in the control group (Figure 7). The *Collinsella* genus was not associated with BMI regardless of *H. pylori* infection status (Figure 8). It has been reported that the *Subdoligranulum* genus is less prevalent among type 2 diabetes patients compared with their non-diabetic counterparts [51], and a negative correlation with insulin resistance has been shown [52]. An increase in the *Collinsella* genus is reportedly associated with increased insulin, triglyceride, and very-low-density lipoprotein levels [53] and is associated with type 2 diabetes [54]. In our study, of the three genera (*Prevotella, Subdoligranylum*, and *Collinsella*) that showed significant differences in relative abundance between the *H. pylori* and control groups, the *Prevotella* genus showed the most significant correlation between *H. pylori* infection status and BMI. The *Prevotella* genus was the only genus that showed an association with BMI in the *H. pylori* group but not the control group.

There are several limitations to the present study. i) In the selection of subjects in both groups, false-negative results by using the urinary antibody in the control group and false-positive results by using stool antigen in the *H. pylori* group could not be completely eliminated. ii) The present study evaluated feces specimens, the microbiota of which may be different from the mucosal-associated microbiota. iii) The effect of eradication of *H. pylori* on the intestinal microbiota could be important [55], and we plan to investigate this in the future. iv) There was a difference in preschool status between the two groups (Table 1), and it could not be completely ruled out that this could have affected the intestinal microbiota.

CONCLUSION

The present study shows that the intestinal microbiota is significantly affected by *H. pylori* infection in junior high school third-grade students in Saga Prefecture, Japan. Furthermore, the relative abundance of the *Prevotella* genus was increased concomitantly with a rise in BMI in *H. pylori*-infected students.

Acknowledgments

We would like to thank Mr. Daisuke Takami of R&D Center, Biofermin Pharmaceutical Co., Ltd. for his co-operation. We thank Ms. Kozue Kakiuchi, Ms. Tomomi Ito, and Ms. Hiromi Beppu for project support.

Competing interests: The authors declare that they have no conflict of interest.

Funding: This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Author contributors

This study was supported by the Biofermin Pharmaceutical Co., Ltd. (Kobe, Japan), performing intestinal microbiota analysis and statistical evaluation. However, their contribution did not influence data analysis or interpretation in this study. The authors (YT and HO) did not play any additional role in the study design, data collection and analysis, publishing decisions, or manuscript preparation.

Study concept and design: TK and KF. Acquisition of data: TK. Analysis and interpretation of data: TK. Drafting of the manuscript: TK. Critical revision of the manuscript for important intellectual content: MM and KF. Statistical analysis: YT and

HO. Administrative, technical, or material support: YT and HO. Study supervision: MM and KF. Writing, reviewing, and editing: MM and KF.

Patients consent for publication: Informed consent was obtained from all individual participants included in the study. Signed informed consent was obtained from each study participant prior to participation in the study.

Ethical approval: The ethical aspects of this study were reviewed and approved by the institutional review board of Saga University Hospital (approval number: 2016-11-03). Written informed consent was obtained from all of the students and their guardians. All methods were carried out in accordance with relevant guidelines and regulations or Helsinki guidelines.

Provenance and peer review: Not commissioned; externally peer-reviewed.

Date availability statement: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

REFERENCES

1 Matsuki T, Yahagi K, Mori H, et al. A key genetic factor for fucosyllactose utilization affects infant gut microbiota development. *Nat Commun* 2016;7:11939.

2 Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. *Nature* 2012;486:222–7.

3 Romero R, Hassan SS, Gajer P, et al. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2014;2:4.

4 Aagaard K, Riehle K, Ma J, et al. A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. *PLoS One* 2012;7:e36466.

5 Backhed F, Roswall J, Peng Y, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 2015;17:690–703.

6 Bezirtzoglou E, Tsiotsias A, Welling GW. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe* 2011;17:478–82.

7 Penders J, Vink C, Driessen C, et al. Quantification of Bifidobacterium spp.,

Escherichia coli and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett* 2005;243:141–7.

8 Bokulich NA, Chung J, Battaglia T, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci Transl Med* 2016;8:343ra82.

9 Mueller NT, Bakacs E, Combellick J, et al. The infant microbiome development: mom matters. *Trends Mol Med* 2015;21:109–17.

10 Fouhy F, Guinane CM, Hussey S, et al. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrob Agents Chemother* 2012;56:5811–20.

11 Tanaka S, Kobayashi T, Songjinda P, et al. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunol Med Microbiol* 2009;56:80–7.

12 Mišak Z, Hojsak I, Homan M. Review: *Helicobacter pylori* in pediatrics. *Helicobacter* 2019;24(Suppl 1):e12639.

13 Brawner KM, Kumar R, Serrano CA, et al. Helicobacter pylori infection is associated with an altered gastric microbiota in children. *Mucosal Immunol* 2017;10:1169–77.

14 Llorca L, Perez-Perez G, Urruzuno P, et al. Characterization of the gastric microbiota in a pediatric population according to *Helicobacter pylori* status. *Pediatr Infect Dis J* 2017;36:173–8.

15 Oh B, Kim BS, Kim JW, et al. The effect of probiotics on gut microbiota during the *Helicobacter pylori* eradication: Randomized controlled trial. *Helicobacter* 2016;21:165–74.

16 Buhling A, Radun D, Muller WA, et al. Influence of anti-Helicobacter triple-therapy with metronidazole, omeprazole and clarithromycin on intestinal microflora. *Aliment Pharmacol Ther* 2001;15:1445–52.

17 Kakiuchi T, Matsuo M, Endo H, et al. A *Helicobacter pylori* screening and treatment program to eliminate gastric cancer among junior high school students in Saga Prefecture: a preliminary report. *J Gastroenterol* 2019;54:699–707.

18 Okuda M, Kamiya S, Booka M, et al. Diagnostic accuracy of urine-based kits for detection of Helicobacter pylori antibody in children. *Pediatrics Int* 2013;55:337–41.

19 Matsuki T, Watanabe K, Fujimoto J, et al. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. *Appl Environ Microbiol* 2004;70:167–73.

20 Fadrosh DW, Ma B, Gajer P, et al. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2014;2:6.

21 Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;7:335–6.

22 Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;26:2460–1.

23 Lozupone C, Knight R. UniFrac: A new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 2005;71:8228–35.

24 Asaka M. A new approach for elimination of gastric cancer deaths in Japan. *Int J Cancer* 2013;132:1272–6.

25 Dash NR, Khoder G, Nada AM, et al. Exploring the impact of Helicobacter pylori on gut microbiome composition. *PLOS ONE* 2019;14:e0218274.

26 Yang YJ, Sheu BS. Metabolic interaction of *Helicobacter pylori* infection and gut microbiota. *Microorganisms* 2016;4.

27 Schulz C, Schutte K, Koch N, et al. The active bacterial assemblages of the upper GI tract in individuals with and without Helicobacter infection. *Gut* 2018;67:216–25.

28 Yin YN, Wang CL, Liu XW, et al. Gastric and duodenum microflora analysis after long-term Helicobacter pylori infection in Mongolian Gerbils. *Helicobacter* 2011;16:389–97.

29 Mitsuoka T, Hayakawa K, Kimura N. The faecal flora of man. II. The composition of bifidobacterium flora of different age groups (author's transl). *Zentralbl Bakteriol Orig A* 1974;226:469–78.

30 Boukthir S, Aouididi F, Mazigh Mrad S, et al. Chronic gastritis in children. *Tunis Med* 2007;85:756–60.

31 Yu Y, Su L, Wang X, et al. Association between Helicobacter pylori infection and pathological changes in the gastric mucosa in Chinese children. *Intern Med* 2014;53:83–8.

32 Hojo M, Asahara T, Nagahara A, et al. Gut microbiota composition before and after use of proton pump inhibitors. *Dig Dis Sci* 2018;63:2940–9.

33 Kinoshita Y, Ishimura N, Ishihara S. Advantages and disadvantages of long-term proton pump inhibitor use. *J Neurogastroenterol Motil* 2018;24:182–96.

34 Mohammadi SO, Yadegar A, Kargar M, et al. The impact of Helicobacter pylori infection on gut microbiota-endocrine system axis; modulation of metabolic

hormone levels and energy homeostasis. *J Diabetes Metab Disord* 2020;19:1855–1861.

35 Benavides-Ward A, Vasquez-Achaya F, Silva-Caso W, et al. Helicobacter pylori and its relationship with variations of gut microbiota in asymptomatic children between 6 and 12 years. *BMC Res Notes* 2018;11:468.

36 Nakayama J, Watanabe K, Jiang J, et al. Diversity in gut bacterial community of school-age children in Asia. *Sci Rep* 2015;5:8397.

37 Lim MY, Rho M, Song YM, et al. Stability of gut enterotypes in Korean monozygotic twins and their association with biomarkers and diet. *Sci Rep* 2014;4:7348.

38 Ley RE, Turnbaugh PJ, Klein S, et al. Microbial ecology: Human gut microbes associated with obesity. *Nature* 2006;444:1022–3.

39 Magne F, Gotteland M, Gauthier L, et al. The Firmicutes/Bacteroidetes Ratio: A relevant marker of gut dysbiosis in obese patients? *Nutrients* 2020;12.

40 Jeon CY, Haan MN, Cheng C, et al. Helicobacter pylori infection is associated with an increased rate of diabetes. *Diabetes Care* 2012;35:520–5.

41 Marietti M, Gasbarrini A, Saracco G, et al. Helicobacter pylori infection and diabetes mellitus: the 2013 state of art. *Panminerva Med* 2013;55:277–81.

42 Furet JP, Kong LC, Tap J, et al. Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: Links with metabolic and low-grade inflammation markers. *Diabetes* 2010;59:3049–57.

43 Moreno-Indias I, Sanchez-Alcoholado L, Garcia-Fuentes E, et al. Insulin resistance is associated with specific gut microbiota in appendix samples from morbidly obese patients. *Am J Transl Res* 2016;8:5672–84.

44 Mouzaki M, Comelli EM, Arendt BM, et al. Intestinal microbiota in patients with nonalcoholic fatty liver disease. *Hepatology* 2013;58:120–7.

45 Roager HM, Licht TR, Poulsen SK, et al. Microbial enterotypes, inferred by the prevotella-to-bacteroides ratio, remained stable during a 6-month randomized controlled diet intervention with the new nordic diet. *Appl Environ Microbiol* 2014;80:1142–9.

46 Fugmann M, Breier M, Rottenkolber M, et al. The stool microbiota of insulin resistant women with recent gestational diabetes, a high risk group for type 2 diabetes. *Sci Rep* 2015;5:13212.

47 Pedersen HK, Gudmundsdottir V, Nielsen HB, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* 2016;535:376–81.

48 Lin L, Wen ZB, Lin DJ, et al. Correlations between microbial communities in

stool and clinical indicators in patients with metabolic syndrome. *World J Clin Cases* 2018;6:54–63.

49 Murri M, Leiva I, Gomez-Zumaquero JM, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. *BMC Med* 2013;11:46.

50 Leiva-Gea I, Sanchez-Alcoholado L, Martin-Tejedor B, et al. Gut microbiota differs in composition and functionality between children with type 1 diabetes and MODY2 and healthy control subjects: A case-control study. *Diabetes Care* 2018;41:2385–95.

51 Khorraminezhad L, Leclercq M, O'Connor S, et al. Dairy product intake modifies gut microbiota composition among hyperinsulinemic individuals. *Eur J Nutr* 2020.

52 Lv Y, Zhao X, Guo W, et al. The relationship between frequently used glucose-lowering agents and gut microbiota in type 2 diabetes mellitus. *J Diabetes Res* 2018;2018:1890978.

53 Gomez-Arango LF, Barrett HL, McIntyre HD, et al. Connections between the gut microbiome and metabolic hormones in early pregnancy in overweight and obese women. *Diabetes* 2016;65:2214–23.

54 Lambeth SM, Carson T, Lowe J, et al. Composition, diversity and abundance of gut microbiome in prediabetes and type 2 diabetes. *J Diabetes Obes* 2015;2:1–7.

55 Takara Y, Endo H, Nakano R, et al. Smoking and drinking did not increase the failure of therapeutic *Helicobacter pylori* eradication by vonoprazan, clarithromycin, and amoxicillin. *Digestion* 2019;99:172–8.

Table 1. Background characteristics of junior high school students in the two groups

		Control group (n = 79)	*H. pylori* group (n = 80)	*P* value
Sex	(male/female)	42/37	46/34	0.80
Age	(years)	14.73 ± 0.33	14.76 ± 0.32	0.71
BMI	(kg/m^2)	19.69 ± 3.48	19.67 ± 2.41	0.97
Delivery	(vaginal/C-section)	68/11	60/11	0.79
Nutrition	(breast/formula/mix)	37/6/36	27/15/36	0.07
School	(nursery/kindergarten/none)	25/54/0	53/25/2	<0.001
Allergies	(+/−)	5/75	7/73	0.55

Delivery: birth delivery style; C-section: cesarean section; nutrition: method of infant nutrition; school: pre-school situation; BMI: body mass index.

Figure legends

Figure 1. Flowchart for *Helicobacter pylori* screening and treatment of junior high school students in Saga Prefecture and the selection method used to establish the two groups.

The *H. pylori* group comprised 80 students, consented to the study, tested positive for both urinary anti-*H. pylori* immunoglobulin-G antibody and stool antigen test. The control group (n = 79) comprised those tested negative for both tests.

Figure 2. Alpha diversity of the 16S rRNA sequences in the control and *H. pylori* groups

The control group exhibited lower microbial species richness compared with the *H. pylori* group. The observed species index (S. obs), chao 1 index, and abundance-based coverage estimator index all showed significantly higher diversity in the *H. pylori* group than in the control group (*$P < 0.001$). The Shannon index was not significantly different between the two groups ($P = 0.054$). OTUs: operational taxonomic units.

Figure 3. Beta diversity of the 16S rRNA/DNA/amplicon sequence data (control group vs *H. pylori* group)

PCO: principal coordinate analysis; PERMDISP: permutational analysis of multivariate dispersions.

Figure 4. The main 13 bacterial types present in the intestinal microbiota at the genus level, comparing the *H. pylori* and control groups.

*$P < 0.05$; **$P < 0.01$.

Figure 5. The seven main bacterial types present in the intestinal microbiota at the genus level for the control group (A) and the *H. pylori* group (B) in association with body mass index (BMI)

*$P < 0.05$; **$P < 0.01$. Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

Figure 6. Relative abundance of the *Prevotella* genus in relation to the BMI category in the *H. pylori* and control groups

Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Figure 7. The ratio of the Firmicutes phylum to the Bacteroides phylum in relation to the BMI category in the *H. pylori* and control groups

Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

***$P < 0.001$.

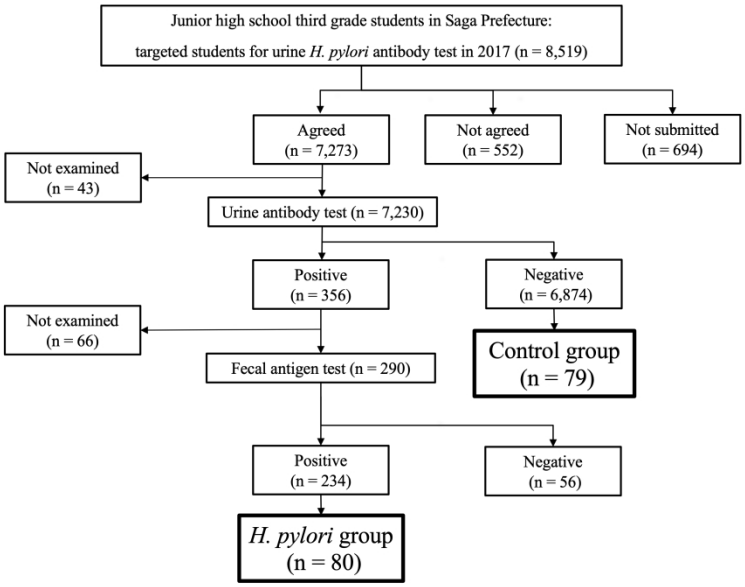
Figure 8. Relative abundance of the *Subdoligranulum* genus in relation to the BMI category in the *H. pylori* and control groups

Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.

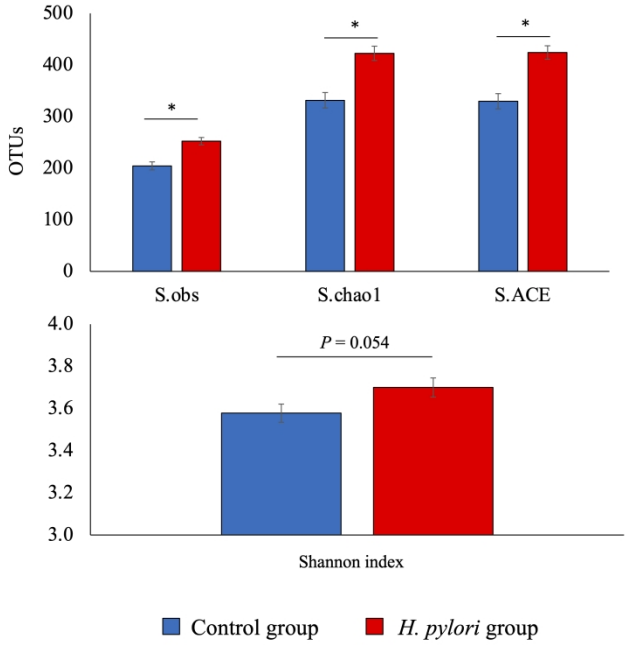
*$P < 0.05$; **$P < 0.01$.

Figure 9. Relative abundance of the *Collinsella* genus in relation to the BMI category in the *H. pylori* and control groups

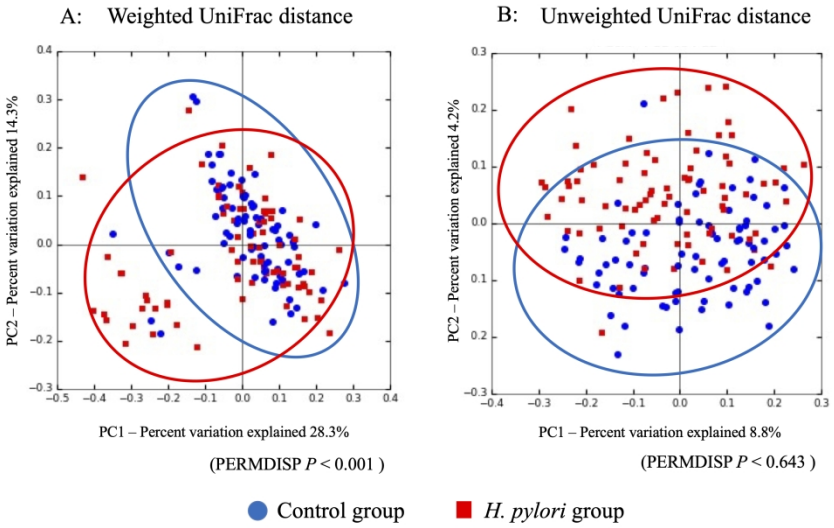
Low: BMI < 15; Mid: BMI of 15–25; High: BMI > 25.



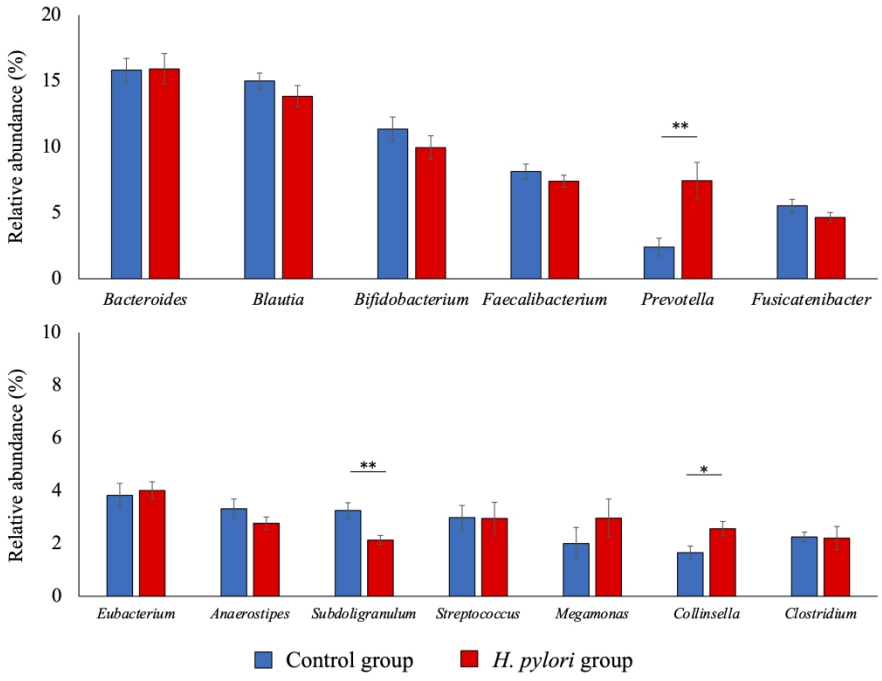
296x209mm (300 x 300 DPI)



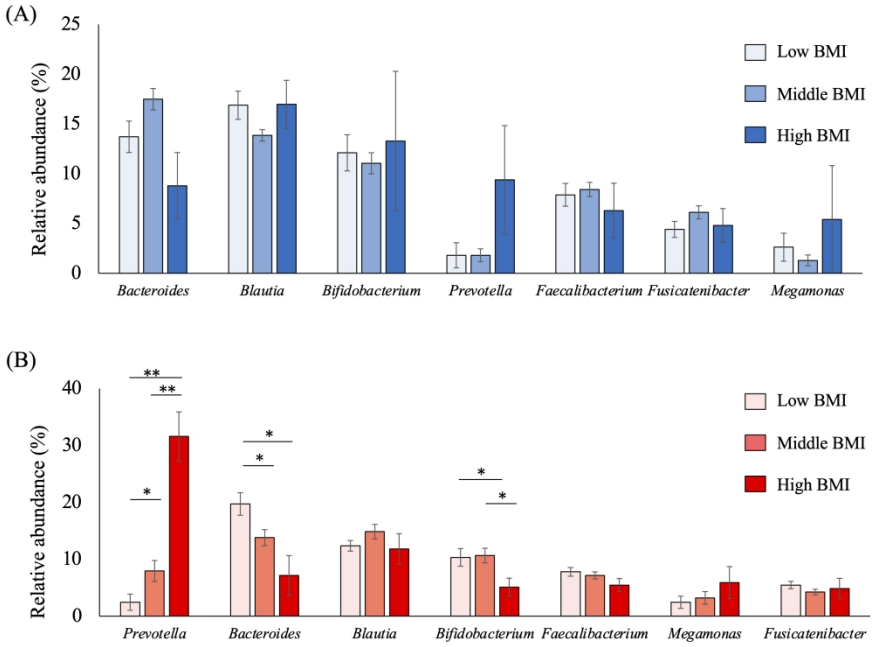
296x209mm (300 x 300 DPI)



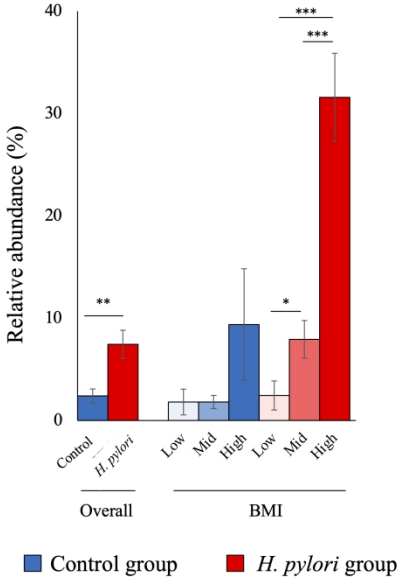
296x209mm (300 x 300 DPI)



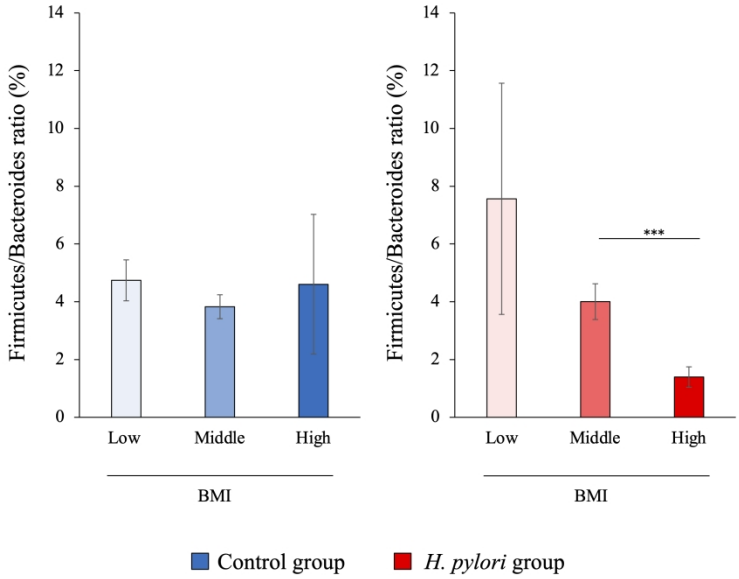
296x209mm (300 x 300 DPI)



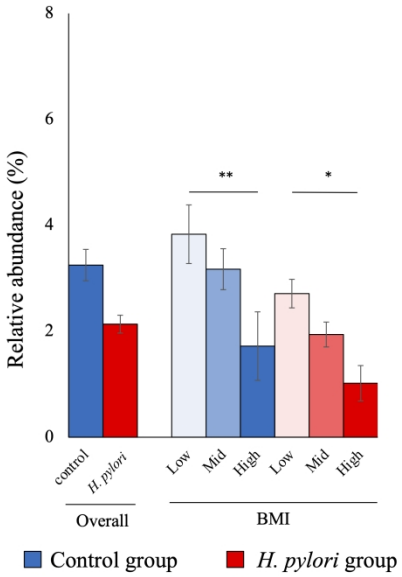
296x209mm (300 x 300 DPI)



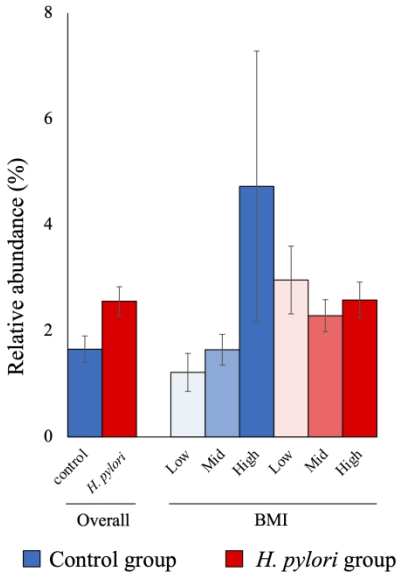
296x209mm (300 x 300 DPI)



296x209mm (300 x 300 DPI)



296x209mm (300 x 300 DPI)



296x209mm (300 x 300 DPI)

Reporting checklist for cross sectional study.

Based on the STROBE cross sectional guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the STROBE cross sectionalreporting guidelines, and cite them as:

von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.

		Reporting Item	Page Number
Title and abstract			
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	1
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	2-3
Introduction			
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	#3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	#4	Present key elements of study design early in the paper	5-6
Setting	#5	Describe the setting, locations, and relevant dates, including periods of	5-6

		recruitment, exposure, follow-up, and data collection	
Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of selection of participants.	6
	#7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-9
Data sources / measurement	#8	For each variable of interest give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group. Give information separately for for exposed and unexposed groups if applicable.	6-9
Bias	#9	Describe any efforts to address potential sources of bias	6-9
Study size	#10	Explain how the study size was arrived at	6-9
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	6-9
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding	9
Statistical methods	#12b	Describe any methods used to examine subgroups and interactions	9
Statistical methods	#12c	Explain how missing data were addressed	9
Statistical methods	#12d	If applicable, describe analytical methods taking account of sampling strategy	9
Statistical methods	#12e	Describe any sensitivity analyses	9
Results			
Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.	10
Participants	#13b	Give reasons for non-participation at each stage	10
Participants	#13c	Consider use of a flow diagram	10

Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	10
Descriptive data	#14b	Indicate number of participants with missing data for each variable of interest	10
Outcome data	#15	Report numbers of outcome events or summary measures. Give information separately for exposed and unexposed groups if applicable.	10-12
Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10-12
Main results	#16b	Report category boundaries when continuous variables were categorized	10-12
Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	10-12
Other analyses	#17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	10-12
Discussion			
Key results	#18	Summarise key results with reference to study objectives	13
Limitations	#19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.	16
Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	13-16
Generalisability	#21	Discuss the generalisability (external validity) of the study results	13-16
Other Information			
Funding	#22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	18